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Identification of a Mutation Cluster in Mevalonate Kinase Deficiency, Including a New Mutation in a Patient of Mennonite Ancestry

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Summary

Mevalonate kinase (MKase) deficiency (MKD) is a rare autosomal recessive disorder in the pathway of cholesterol and nonsterol isoprenoid biosynthesis. Thus far, two disease-causing missense alleles have been identified, N301T and A334T. We report four additional mutations associated with MKD: L264F, T243I, L265P, and I268T, the last found in a patient of Mennonite ancestry. Electrophoretic analysis of bacterially expressed wild-type and mutant MKase indicated that I268T and T243I mutants produced normal or somewhat reduced amounts of MKase protein; conversely, L264F and L265P mutations resulted in considerably decreased, or absent, MKase protein. Immunoblot analysis of MKase from all patients suggested that the MKase polypeptide was grossly intact and produced in amounts comparable to control levels. Three mutations resulted in significantly diminished MKase enzyme activity (<2%), whereas the I268T allele yielded ~20% residual enzyme activity. Our results should allow more-accurate identification of carriers and indicate a mutation "cluster" within amino acids 240–270 of the mature MKase polypeptide.

Introduction

Mevalonate kinase (MKase; MIM 251170; E.C.2.7.1.36) deficiency (MKD) is an inborn error of metabolism in the early region of the cholesterol and nonsterol isoprenoid biosynthetic pathway (Hoffmann et al. 1993). MKase phosphorylates 3,*R*-mevalonic acid to produce 5-phosphomevalonic acid, a pivotal compound in cholesterol and isoprenoid synthesis. In addition to cholesterol, subsequent steps in the metabolism of mevalonate generate isopentenyl tRNA, dolichol, ubiquinones, and Heme A, as well as farnesylated and geranylgeranylated proteins. Because these intermediates have critical roles in intermediary metabolism, information on the production and disposition of 5-phosphomevalonic acid is of interest.

The pathology of MKD is multisystemic, with considerable phenotypic variability. In 19 patients, clinical findings include mild-to-moderate psychomotor retardation, failure to thrive, hypotonia, ataxia, anemia, hepatosplenomegaly, dysmorphic features, cataracts, lymphadenopathy, myopathy, and fat malabsorptive enteropathy. The course of the disease has been fatal for about half of these patients, whereas others have reached late adolescence or early adulthood.

The highly regulated reaction prior to MKase is catalyzed by 3-hydroxy-3-methylglutaryl (HMG) CoA reductase. A biochemical hallmark of MKD is significantly increased levels of mevalonic acid in urine, 0.9–56 mol/mol creatinine (normal < 0.003 mol/mol creatinine). HMG CoA reductase is up-regulated in patients with MKD and cannot be completely suppressed even with the administration of potent HMG-CoA reductase inhibitors. Although the accumulation of mevalonic acid in tissues is likely to contribute to disease pathophysiology, patients have decompensated when given drugs whose pharmacologic action is inhibition of HMG-CoA reductase (Hoffmann et al. 1993). This may indicate that overaccumulation of mevalonic acid could ameliorate some aspects of the symptoms (Gibson et al. 1990).

To elucidate the underlying mechanisms associated with MKD, we have undertaken molecular genetic analyses in affected individuals. The human MKase cDNA

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was cloned, and the first disease-linked mutation in the index patient (an asparagine to threonine substitution at position 301) was identified (Hoffmann et al. 1986; Schafer et al. 1992). More recently, we identified an alanine to threonine substitution at amino acid 334 that significantly altered *R*,*S*-mevalonate binding (Hinson et al. 1997*a*). We now present four new mutations in six patients (from four families) with MKD. We have identified the second disease-associated mutation in the index patient as an isoleucine to threonine substitution at position 268 (I268T), which was also detected as one allele in a patient of Mennonite ancestry. Although Mennonite ancestry is not acknowledged in the family of the index patient, the surname of the maternal side of the family is closely linked to the Mennonite community. To verify that the I268T allele was disease linked, we performed extensive pedigree analyses by using enzymatic and sequencing methods to determine the transmission of this allele in the family of the index patient. The three remaining mutations clustered in close proximity to this region at amino acids 243, 264, and 265. Bacterial-expression analysis and subsequent enzymatic analysis verified the disease-causing nature of these mutations. The present report summarizes our findings.

Material and Methods

Material

Material was purchased from manufacturers as follows: custom synthesized gel-purified oligonucleotides, chemically competent *Escherichia coli* DH5α, Moloney murine leukemia virus (MMLV) reverse transcriptase, deoxynucleotides, and T4 DNA ligase (Gibco-BRL); Sequenase DNA sequencing kit, Thermosequenase sequencing kit (Amersham/U.S. Biochemical); Expand* DNA polymerase, restriction endonucleases, and RNase inhibitor (Boehringer Mannheim); α-^{35}S-dATP (58 mCi/mmol) and ^{33}P-ddNTPs (Amersham/Lifescience); Kodak Biomax film, VWR; Qiaex II DNA isolation kit and *Taq* polymerase (Qiagen); OptiMem tissue culture media (Life Technologies); and expression plasmid pGEX 4T-3 and GST purification kit (Pharmacia). All other reagents were of the highest purity available. The use of cultured cells derived from patients was submitted and approved by the institutional review board on human subjects of the Baylor University Health Care Campus, Dallas. Consent from all family members of patient 1 was obtained prior to the collection of blood specimens.

RNA Extraction

Patient lymphoblasts or fibroblasts were grown in RPMI media supplemented with 20% FCS , l-glutamine, and streptomycin/kanamycin until confluent. The cells were harvested by centrifugation and washed three times at room temperature with PBS. The supernatant was decanted and the cell pellet used immediately or stored at −80°C. RNA was extracted by adding 1–3 ml phenol, 0.1–0.3 ml 2 M sodium acetate (pH 4), and 1–3 ml of a solution composed of 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7), and 7.5 μM β-mercaptoethanol to each pellet. The cells were resuspended in this solution and passed through a syringe fitted with a 21–25-gauge needle to shear genomic DNA. The suspension was divided into 1-ml aliquots, and 0.1 ml chloroform was added to each. The samples were mixed and incubated on ice for 15–30 min. The samples were centrifuged at 12,000 *g* and 4°C for 20 min. The aqueous phase was transferred to a 1.5-ml microcentrifuge tube and 0.5 ml isopropanol was added. The samples were precipitated at −20°C for 1–24 h. The RNA was pelleted by centrifugation for 30 min at 12,000*g* and washed with 70% ethanol. The pellet was dried and resuspended in 50–100 μl H_2O.

Reverse Transcription-PCR for Sequence and Cloning Analysis

First-strand cDNA was synthesized by incubation of 5–10 μg RNA with 27.5 μl H_2O at 90°C for 5–10 min to denature RNA secondary structure. Samples were quick chilled on ice for 2 min. The samples were centrifuged briefly. Two microliters of MMLV reverse transcriptase, 0.5 μl 0.1 M DTT, 10 μl first-strand RT buffer, 2 μl 10-mM nucleotides, 0.5 μl RNase inhibitor, and 20 pmol MK5 primer were added to each and were incubated at 42°C for 1–2 h. MK5, a downstream gene-specific antisense primer, had the following sequence: 5′-GGCTCGAGCACAGAGTCGAACTGCAGCC-3′. The samples were ethanol precipitated for 1–24 h at −20°C with 0.1 vol 3-M sodium acetate (pH 4) and 2 vol absolute ethanol. The cDNA was pelleted by centrifugation for 10 min at 12,000 rpm and was washed with 70% ethanol. The samples were dried and resuspended in 80 μl H_2O.

Two sets of PCR were performed with two sets of primers to overlap the MKase cDNA. MK4 and MK2 primers were used to amplify 25 nucleotides of the 5′-untranslated region of the cDNA, with extension through nucleotide 993 of the coding region. MK1 and MK3 primers were used to amplify nucleotides 468–1220, with the stop codon ending at nucleotide 1191. The MK4 primer contained an *Eco*RI site on the 5′ end with the sequence: 5′-GGGAATTCAGGATTC-CCAGGAGCCATG-3′. MK2 was an antisense downstream primer with the sequence: 5′-CAGCTTGCTG-TGAAGTCCGCGG-3′. MK1 was a sense primer with the sequence: 5′-GGATCCCTCCTGACTGTGTGCAG-3′. MK3 was a antisense downstream primer containing a *Xho*I restriction site on the 3′ end with the sequence:

5′-GGCTCGAGGCTGCAGTGTCGTGGGCTCCTCT-CAG-3′.

One-eighth of the RT reaction was used in each PCR reaction. The following were added to each reaction mixture: 5 μl 10X PCR–reaction buffer containing 1.5 mM Mg_2Cl, 10 pmol each primer, and 0.5 μl 10-mM nucleotides, in a final volume of 50 μl. To prevent amplification of non-MKase cDNA, 1.7 U EXPAND polymerase was not added until the first annealing step of PCR. Forty cycles of PCR were performed on the cDNA, with the following profile: 4-min hot start at 95°C; step 1, 94°C for 1 min; step 2, 57°C for 1 min; step 3, 72°C for 1 min; step 4 for 40 cycles, followed by step 5, 72°C for 5 min; and step 6, 4°C. A 15–20-μl aliquot of the PCR reaction was analyzed on a 0.7% agarose gel. The correctly sized PCR products (1,020 and 752 bp, respectively) were excised and extensively purified with the QiaEx II DNA purification kit.

Both sets of PCR products were directly sequenced and compared with the wild-type sequence in a search for disease-linked alterations. If a mutation was detected, the PCR product containing that mutation was cassette-ligated into the wild-type MKase cDNA in the expression vector pGEX 4T-3, after excision of the wild-type fragment.

PCR Amplification of the MKase cDNA for MvaI *Digestion*

One mutation that we identified, the threonine to isoleucine substitution at amino acid 243 (discussed later) was further analyzed in patient and parents by using restriction digestion with *Mva*I. An additional PCR was done to produce a 714-bp band for digestion with this restriction endonuclease. The primers used were sense, 5′-CTGGATATCGTAGTGTGGTCGGA-3′ and antisense, 5′-GTCAAAGCCACAGCTGGTCA-G-3′. This 714-bp product contained nucleotide 728 and only seven of the 12 *Mva*I sites within the MKase-coding region. Digestion of this product provided a readily distinguishable pattern for the T243I mutation at nucleotide 728 (C→T substitution).

Cloning, Sequencing, and MvaI *Digestion*

Chemically competent *E. coli* DH5α cells were used for transformation. Clones were completely sequenced to verify that the mutation of interest was the only mutation introduced. Sequencing was done with the Thermosequenase kit (Amersham) according to the manufacturer's protocols. pGEX vector primers, as well as internal primers, were used for sequencing. The C→T mutation at nucleotide 728 resulted in the loss of one *Mva*I restriction site. Purified PCR products, derived from patient 2 (table 1) and the parents, were digested with 20 U *Mva*I at 37°C for 2 h and analyzed on a 3% (3:1) agarose gel (NuSieve).

Table 1

Mutations Identified in Cultured Cells Derived from MKD Patients and Residual Activity of the Corresponding Recombinant Glutathione *S*-Transferase Fusion Proteins

Patient and Alleles[a]	Fusion Protein Enzyme Activity[b]
1:	
I268T	2.43 ± .41 (n = 9)
N301T	.19 ± .04 (n = 4)
2:	
T243I	.20 ± .04 (n = 3)
L265P	None detected (n = 3)
3a–c:[c]	
L264F	.04 ± .001 (n = 4)
Allele 2	Unknown
4:	
I268T	2.43 ± .41 (n = 9)
Allele 2	Unknown
5a and *5b:*[c]	
A334T	.26 ± .02 (n = 6)[d]
Control	13.0 ± 1.1 (n = 30)[e]

[a] Clinical details have been reported for patient 1 (Hoffmann et al. 1986), patient 2 (Kozich et al. 1991)m patients *3a–c* (Mancini et al. 1993), patient *5a*, and patient *5b* (Hoffman et al. 1993). Alleles refer to the corresponding amino acid of the polypeptide, with wild-type amino acid on the left and resulting mutant amino acid on the right (single letter designation).

[b] The letter "n" denotes number of determinations on different days; all values presented are mean ± SEM (standard error of the mean).

[c] Patients *5a* and *5b* are siblings. Preliminary results indicate that two additional unrelated siblings also have the L264F allele in a homozygous pattern (J. L. Shaw and K. M. Gibson, unpublished data).

[d] See Hinson et al. (1997*a*).

[e] Range 7.0–32.8 μmol/min/mg protein.

Protein Expression, Enzyme Assay, SDS-PAGE, and Protein Alignment

Expression of mutant cDNAs was used to assess the effect of identified mutations on MKase enzyme activity. Expression was performed with the glutathione *S*-transferase fusion vector pGEX 4T-3 system (Pharmacia) (Chambliss et al. 1995, 1996). The recombinant-fusion proteins were purified with glutathione Sepharose beads, as described by the Pharmacia GST-fusion batch-purification protocol, and enzyme activity was determined immediately after purification.

MKase activity for purified recombinant enzymes was determined with use of a standard spectrophotometric assay, described elsewhere by Hinson et al. (1997*b*). To determine MKase enzyme activity in extracts of lymphocytes isolated from whole blood, a radiometric assay was used as described elsewhere by Gibson et al. (1989).

As control for tissue viability in lymphocyte preparations, propionyl-coenzyme A carboxylase (PCC) was assayed with use of a standard ^{14}C-bicarbonate fixation assay, with propionyl-coenzyme A as substrate (Gibson et al. 1996). Purified wild-type and mutant recombinant proteins were analyzed on a 10% SDS-polyacrylamide gel with visualization by Coomassie staining, to determine the effect of inherited mutations on protein production. Protein alignments were performed by use of the Hitachi MacDNAsis (v. 3.6).

Immunoblotting

SDS-PAGE and immunoblotting were performed as described elsewhere by Biardi et al. (1994), with the following modifications: cell extracts from cultured lymphoblasts and fibroblasts of patients were obtained by homogenization in buffer (50 mM KH_2PO_4, 30 mM EDTA, 200 mM NaCl, and 10 mM DTT [pH 7.0]) supplemented with 0.2% triton X-100. Cellular debris was removed by centrifugation. Protein concentrations were determined by use of a BCA kit (Pierce), with BSA as standard.

For western blotting, ~0.2 mg protein per sample was electrophoresed on a 10% SDS-polyacrylamide gel. The primary antibody was a polyclonal antipeptide antibody produced in rabbit against the C-terminal region of MKase, as described elsewhere (Biardi et al. 1994). The secondary antibody was a protein A-HRP conjugate (Bio-Rad). Washes were performed in phosphate-buffered saline tween (0.02 M KH_2PO_4, 0.5 M NaCl, and 0.05 M Tween 20), with a total of three washes for 5 min each after incubation of the primary antibody, and three washes at 30 min each after incubation with secondary antibody. Immunoreactive bands were visualized with the ECL kit (New England Nuclear).

Results

Clinical Findings in Patients

Clinical summaries have been presented on all patients described in this report except patient 4, who has not been reported. In brief, patient 1 presented with severe failure to thrive, developmental delay, anemia, hepatosplenomegaly, central cataracts, and dysmorphic features (Hoffmann et al. 1986). Patient 2 suffered severe psychomotor retardation, hypotonia, tremor, ataxia, nystagmus, failure to thrive, and agenesis of the vermis cerebelli with cerebellar atrophy (Kozich et al. 1991). Patients 3*a* and 3*b* were two of a three-patient sibship. All three siblings manifested failure to thrive, susceptibility to infections, hepatosplenomegaly, cataracts, and psychomotor retardation. Dysmorphic features were apparent in two of three sibs (Mancini et al. 1993). Patients 5*a* and 5*b* (siblings, male and female) manifested recurrent febrile crises, hypotonia, failure to thrive, ataxia, cerebellar atrophy, and psychomotor retardation.

Identification and Inheritance of an I268T Mutation in Patient 1 and 4

A T→C mutation was identified at nucleotide 803 by direct sequencing of amplified cDNAs derived from two MKD patients (patient 1 and 4) in different families (table 1). This mutation resulted in substitution of threonine for isoleucine at amino acid 268 (I268T). DNA sequencing revealed that patient 4, who is of Mennonite descent, was a compound heterozygote, with the I268T mutation as one disease-associated allele. The father of patient 4 carried the I268T allele, and the mother had wild-type sequence at this position (data not shown). Direct sequencing revealed that the index MKD patient (patient 1) also harbored the I268T allele, confirming him as a compound heterozygote of I268T and N301T mutations (table 1). Direct sequencing of amplified cDNAs revealed that the mother of patient 1 contributed the I268T allele, whereas the father had wild-type sequence at this position.

Because the cDNA sequence of MKase is GC-rich, significant compressions could occur during DNA-sequence analysis. Since the I268T allele had not been detected in the mother of patient 1 in our earlier study of this family (Schafer et al. 1992), we sought to verify that the I268T allele was not simply a common polymorphism. Moreover, because this mutation was detected in a patient of Mennonite ancestry, and it is probable that we may again encounter the mutation in this population, it was important to verify the causative nature. The maternal side of the family of patient 1 was amenable to DNA and enzyme analysis, and 30 blood specimens were obtained for verification of the I268T allele in this cohort (fig. 1). Comparison of enzyme analyses and direct DNA sequencing, depicted in figure 1, showed very good agreement for 11 of 12 family members from the maternal side of the family of patient 1. Although the results of enzyme studies for the maternal great-grandfather were unclear, direct sequence analysis verified that he carried the I268T allele. Two of the family members were known carriers of the previously identified N301T mutation (fig. 1) and thus served as negative controls (i.e., individuals with intermediate enzyme activity), whereas three other individuals served as positive controls (i.e., individuals expected to display wild-type levels of enzyme activity) having entered the family through marriage (Goebel-Schreiner et al. 1995).

The family of patient 1 does not acknowledge any known Mennonite ancestry, although they live in a community with a considerable Mennonite Amish population. In addition, the maternal great-grandfather of patient 1 has the same surname as the Mennonite patient

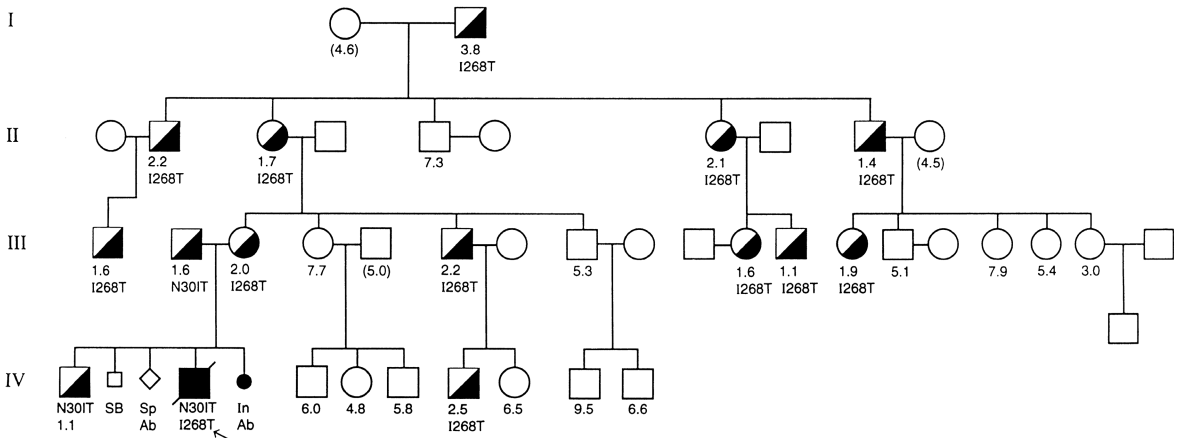

Figure 1 Maternal pedigree of the index family with MKD. White cells were isolated from whole blood for I268T mutation screening in conjunction with MKase enzyme assay. Sequencing and enzyme determinations represent the mean of at least duplicate determinations. Numeric values represent the mean ratio of MKase activity to the marker enzyme, PCC. The ratio is used to correct for variation in white-cell isolation, sonication, and other mechanical manipulations. The proband is indicated with the arrow. His father and brother are carriers of the N301T mutation and serve as internal controls ($n = 2$) for carrier detection by enzyme assay. Individuals whose enzyme ratio is depicted in parentheses were not family related and thus served as positive controls ($n = 3$) for enzyme analyses. N301T = asparagine to threonine mutation at amino acid 301; I268T = isoleucine to threonine substitution at amino acid 268; InAb = induced abortion; SpAb = spontaneous abortion; SB = stillbirth. The proband is a compound heterozygote of N301T/I268T mutations.

4 in our studies. We are currently looking for nearby polymorphic markers to test this further by haplotype analysis.

Identification and Inheritance of T243I and L265P Mutations in Patient 2

Sequencing of amplified cDNAs derived from cultured cells of patient 2 revealed two putative disease-linked mutations. A nucleotide 728 C→T mutation converted threonine to isoleucine (T243I), and a nucleotide 794 T→C mutation resulted in substitution of proline for leucine at position 265 of the polypeptide sequence (L265P) (table 1). The T243I allele was amenable to restriction-digestion analysis, as the nucleotide substitution resulted in the loss of one *Mva*I restriction site. After PCR amplification for restriction digestion (see Material and Methods), analysis with *Mva*I verified that the father of patient 2 contributed the T243I allele, whereas the mother had wild-type sequence and a normal *Mva*I digest pattern (fig. 2). Direct sequencing of cDNAs derived from cultured cells of the parents of patient 2 revealed the C→T conversion at nucleotide 728 in the father and the T→C substitution at nucleotide 794 in the mother (data not shown).

Identification of an L264F Mutation in Three Siblings (Patients 3a, 3b, and 3c)

Sequencing of amplified cDNAs derived from cultured cells of three affected siblings (table 1) revealed a C→A

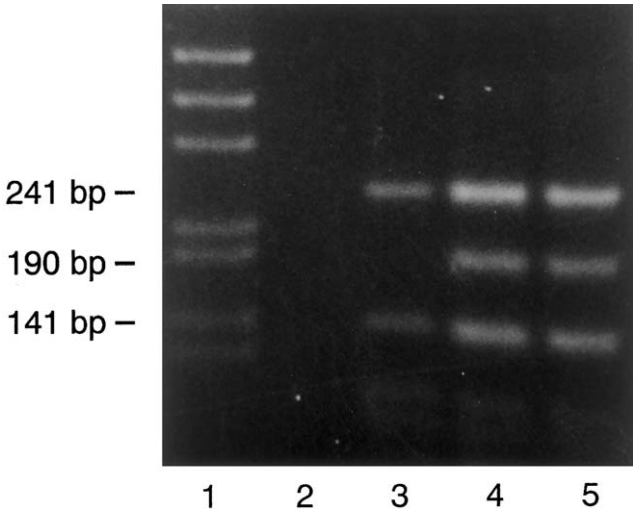

Figure 2 *Mva*I restriction digestion of cDNAs derived from cultured cells of patient 2 and parents for analysis of the T243I allele. The size of the original PCR-derived amplicon was 714 bp, as described in Material and Methods. Restriction digestion of this amplicon results in 7 products at 241, 141, 102, 88, 71, 62, and 8 bp, respectively, corresponding to 6 *Mva*I sites. The T243I allele results in the loss of an *Mva*I site, creating a new product at 190 bp. Heterozygosity for the T243I allele produces a 190-bp band along with the wild-type pattern. Lane contents of the gel were as follows: *lane 1*, DNA molecular-weight standard; *lane 2*, blank; *lane 3*, mother of patient 2; *lane 4*, father of patient 2; *lane 5*, patient 2.

transversion at nucleotide 790 resulting in a substitution of phenylalanine for leucine at amino acid 264 (L264F). Parents were unavailable for testing, and a second disease-linked allele has not been detected. We hypothesized that this allele was deleterious because it was not detected in >50 control samples and because it occurred in a region of conservation for a branched-chain amino acid (fig. 4). We have recently identified the L264F allele in two affected male siblings, inherited in a homozygous pattern (J. L. Shaw and K. M. Gibson, unpublished data).

Enzyme Assay, SDS-PAGE, and Protein Alignment of Purified Recombinant Proteins

Recombinant MKase proteins were assayed spectrophotometrically (Hinson et al. 1997*b*). SDS-PAGE analysis of the purified recombinant-fusion proteins with glutathione *S*-transferase is shown in figure 3. The deduced M_r of the fusion protein with glutathione *S*-transferase is ~74 kDa. As shown in figure 3, only the protein containing the T243I mutation produced a normal amount of fusion protein, with wild-type fusion protein in lanes 1 and 4 for comparison. The protein containing the I268T allele had a reduced amount of protein (<50% of control), whereas the protein containing the L264F mutation had markedly reduced protein (<10% of control), which presented as a doublet on the gel. Lowered amounts of protein and altered product sizes suggested enhanced protein lability, at least in extracts of *E. coli*. The L265P mutation resulted in almost complete absence of detectable MKase fusion protein. Of the four mutations identified, only the I268T protein displayed modest amounts of residual MKase activity (~20% of wild-type activity; the remaining three mutations resulted in enzyme preparations with <2% residual enzyme activity [table 1]).

Immunoblot Analysis of MKase in Cultured Cells Derived from Patients

To assess the effect of mutations on protein stability in cultured cells from patients, immunoblot (western) analysis was performed. As shown in figure 5, cell extracts for all patients showed amounts of MKase protein (~M_r 42 kDa) that were comparable to control. For comparison with table 1 and figure 3, the lane contents in figure 5 were as follows: lane 1, patient 1 (see table 1); lane 2, patient 2; lane 3, patient 4 (see table 1); lane 4 and 5, patient 3*a* and 3*b* (see table 1; patient 3*c* was not analyzed); and lane 6, control lymphoblast-cell extract. Whereas the results for the T243I allele were consistent when expression in *E. coli* and immunoblot analysis was compared, the findings for alleles L265P, L264F, and I268T were discordant in the two methods of analysis.

Discussion

We report four disease-causing mutations responsible for MKD. Identification of a mutation in the Mennonite population may provide a useful tool for detection of heterozygotes in Mennonites, a population in which founder effects for rare disease is well documented (for example, maple-syrup urine disease). The observation that the one Mennonite patient and the "putative" Mennonite patient were both compound heterozygotes argues against the I268T allele being a main mutation. The I268T mutation had been overlooked in our earlier study in which the N301T mutation had been identified. Because of the presence of this allele in the Mennonite population, we wanted to verify that the I268T mutation was functionally deleterious.

I268T results in a significant decrease in MKase activity (to 20% of control values) upon bacterial expression (table 1). This allele was followed-up through four generations (30 individuals) on the maternal side of the index patient's family, with close association between its presence and individuals with intermediate levels of leucocyte MKase enzyme activity (fig. 1). Enzymatic analysis alone represents an imprecise method for determination of the heterozygous condition; thus, we used enzymatic methods in conjunction with direct DNA sequencing to verify the I268T mutation, with good correlation between the two techniques (fig. 1). Recombinant I268T-containing MKase had considerably higher residual activity than the other three mutations presented in this report (table 1). Although the second allele is currently unknown in patient 4, heterozygotes carrying the N301T allele often displayed <50% residual MKase enzyme activity, suggesting that the N301T is a dominant-negative mutation (Gibson et al. 1989).

I268T was found only in the heterozygous condition in our studies, which suggested the possibility that I268T homozygotes could be asymptomatic with sufficient residual activity for pathway function. Thus, for our two patients, symptoms would be expected only when the I268T allele was coupled with a null mutation (i.e., in the case of the index patient, the N301T allele). However, this hypothesis is not supported by the recent detection of an apparently unrelated patient who is homozygous for the I268T mutation and presents with severe symptoms and almost complete MKD (Houten et al. 1998). Nonetheless, it will be of interest to determine the incidence of the I268T allele in the Mennonite population, especially since this allele is appearing to be a somewhat more common mutation.

The four mutations described in the current report bring the total number of mutations identified in MKD to six (Schafer et al. 1992; Hinson et al. 1997*a*). A number of interesting points about MKD can be inferred from our studies. With the exception of two previously

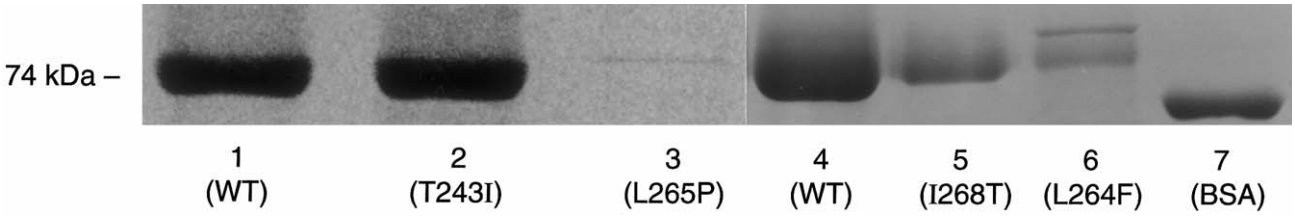

Figure 3 SDS-PAGE analysis of wild-type and mutant recombinant MKase proteins after bacterial expression and purification on glutathione Sepharose beads. The size of the fusion protein of MKase with glutathione *S*-transferase is 74 kDa. Lane contents are as follows: *lane 1,* wild type; *lane 2,* T243I mutant; *lane 3,* L265P mutant; *lane 4,* wild type; *lane 5,* I268T mutant; *lane 6,* L264F mutant; and *lane 7,* BSA (molecular weight 68 kDa), as control. Each lane contained 8–12 μg total purified protein extract isolated from bacterial sonicates. For lanes 3, 5, and 6 the majority of protein was unfused glutathione *S*-transferase, which electrophoresed at an M_r of 31 kDa near the bottom of the gel (not shown). For enzyme-specific–activity estimations, only the area of the band at M_r 74 kDa, determined by densitometric scanning, was used to determine the final residual MKase specific activity for each preparation.

reported siblings, all patients appear to be compound heterozygotes, suggesting the inheritance of very rare alleles in the human population. This is consistent with the low number of patients that have been thus far identified. The latter observation is further supported by the inheritance of the I268T allele, which is only one of two alleles in the Mennonite patient and also in the patient in whom ancestry infers a Mennonite background. Haplotype analysis is currently underway in patient 1 and 4 to determine whether they inherited the same mutant allele or if there were two independently-inherited mutations.

Close analysis of table 1 indicates that of six reported mutations only one (A334T) involves a CpG dinucleotide, which contrasts with the observation that ~30% of human mutations occur in CpG doublets. This finding is also odd in view of the fact that MKase is a relatively GC-rich gene. Although the patient number still remains quite low, this observation may be explained by ascertainment bias, since many MKase-deficient patients may escape detection, or the disease may be lethal in utero. Conversely, the mutation frequency may be very low, or the gene is hypomethylated such that mutations in CpG dinucleotides are infrequent. In any event, analysis of additional patients will provide insight into these questions.

	239	254	268
human	VPRN**T**RALVAGVRNR	L-LKFPEIVAP**LL**TS	**I**DAISLECERVLGEM
rat	VPRS**T**KALVAGVRSR	L-IKFPEIMAP**LL**TS	**I**DAISLECERVLGEM
A. Thal	VGRN**T**KALVSGVSQR	A-VRHPKAMKS**VF**NA	**V**DSISKELAAIIQSK
S.pombe	QPKS**T**KKLVQGVFEL	K-ERLPTVIDS**II**DA	**I**DGISKSAVLALTS-
S.cer	IPRS**T**KDLVARVRVL	VTEKFPEVMKP**IL**DA	**M**GECALQGLEIMTKL
M.thermo	YSGE**T**ARMVAGVAER	F-RRFPDIMGR**IM**DT	**V**ESITNTAYRELLR-

		289	304
human	---------GEAPAP	EQYLVLEELIDM**N**QH	HLNALGVGHASLDQL
rat	---------AAAPVP	EQULVLEELMDM**N**QH	HLNALGVGHASLDQL
A. Thal	---------DETSVT	EKEERIKELMEM**N**QG	LLLSMGVSHSSIEAV
S.pombe	----------ESDKN	SSAKKLGEFIVL**N**QK	LLECLGVSHYSIDRV
S.cer	SKCKGTDDEAVETNN	ELYEQLLELIRI**N**HG	LLVSIGVSHPGLELI
M.thermo	------------NNT	EP---LGELMNL**N**OG	LLDSMGVSTRELSMM

Figure 4 Alignment of MKase amino acid sequence from six species. Amino acids 239–318 are shown. Numbering corresponds to human amino acid sequence. Bold-type letters indicate inherited mutations identified in the current report, in addition to the original N301T allele. A. Thal = *A. thaliana;* S. pombe = *S. pombe;* S. cer. = *S. cerevisiae;* M. thermo = *Mycobacterium thermoautotrophicus*. To aid in alignment between species, gaps have been introduced (dashes). The A334T mutation (table 1) is not shown.

To understand the effect of inherited mutations on protein function, especially the association of the high residual-enzyme activity associated with the I268T allele and the severe disease it causes, it may be useful to consider the biology of MKase. MKase, an extremely hydrophobic homodimeric protein, historically has been considered a cytosolic protein, although recent evidence suggests a peroxisomal location (Tanaka et al. 1990; Biardi et al. 1994). MKase contains a consensus-peroxisome–targeting sequence (PTS), located in the N-terminal region, but there have been no reports to date demonstrating targeting to peroxisomes (Biardi et al. 1994). Because none of our mutations affect this PTS, it is unlikely that putative peroxisomal targeting is disrupted in the disease state. It remains possible that MKase might be membrane-limited, perhaps enabling certain mutations to result in abnormal localization more than abnormal intrinsic (decreased) activity of the enzyme. This might be the case for the I268T allele.

Immunoblotting of MKase from cultured cells of patients, under denaturing conditions, indicated that MKase was grossly intact and produced in levels comparable to control (fig. 5). While these data corroborated those for the T243I allele expressed in *E. coli*, they were discordant with the data of figure 3 for alleles I268T, L264F and L265P, in which expression of the fusion proteins between MKase and glutathione *S*-transferase resulted in moderately to significantly decreased production of recombinant protein. In previous work, Tanaka and coworkers transiently expressed recombinant MKase from patient 1, the index patient, using COS-7 cells (Schafer et al. 1992). In those studies, the amount

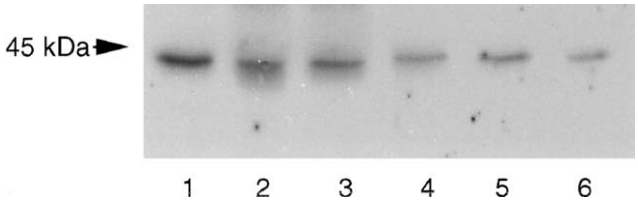

Figure 5 Immunoblot analysis of cell extracts derived from cultured fibroblasts or lymphoblasts of patient 1, 2, 3*a* 3*b*, and 4 (table 1). The primary antibody was an antipeptide antibody raised in rabbit against the C-terminal region of the MKase polypeptide. For comparison with figure 3 and table 1, lane contents were as follows: *lane 1*, patient 1 (alleles N301T and I268T); *lane 2*, patient 2 (alleles L265P and T243I); *lane 3*, patient 4 (allele I268T and second allele unknown); *lane 4 and 5*, patient 3*a* and 3*b* (allele L264F and second allele unknown); and *lane 6*, control.

of MKase protein for patient 1, detected by immunoblot of the expressed recombinant protein, was comparable to controls although residual enzyme activity was ~17% of wild type. These data are consistent with our immunoblot analyses of the same patient and suggest that the decreased amount of protein for I268T, L264F, and L265P proteins may relate specifically to material expressed from *E. coli*. Because all of these analyses were performed under denaturing conditions, we cannot rule out the possibility that subunit interactions are altered by some (or all) of the mutations we have identified. However, our immunoblot data suggests that the gross tertiary structure of the MKase protein in patient cell lines is intact, suggesting that decreased enzyme activity may relate to alterations in the enzyme active-site microenvironment. Some of these questions will be addressed more fully when the crystal structure of the MKase protein is completed.

Sequence alignment of MKase from six species (fig. 4) reveals that three of four mutations reported here involve relatively well-conserved branched-chain amino acids in a mutation cluster. As shown in figure 4, branched-chain amino acids (leucine, isoleucine, or valine) occupy 15 of 18 positions corresponding to amino acids 264, 265, and 268 of the human polypeptide. When there is no branched-chain amino acid in three species, hydrophobic amino acids (methionine or phenylalanine) substitute. Our finding that substitution of these hydrophobic amino acids with other amino acids (proline or threonine) results in decreased MKase activity indicates that hydrophobicity within this region may be important for enzyme function or dimer association. This is also consistent with the high index of hydropathy for rat and human MKase, a surprisingly hydrophobic protein considering that it is predicted to be soluble or peroxisomally located (Tanaka et al. 1990; Biardi et al. 1994; Hinson et al. 1997*a*).

Figure 4 shows an absolute conservation of threonine 243 and asparagine 301 between species. Isoleucine 268 is conserved between human, rat, and *Schizosaccharomyces pombe*, whereas leucine 264 is conserved between human and rat. Leucine 265 is conserved between human, rat, and *Saccharomyces cerevisiae*. None of these amino acids associated with inherited defects in MKD lie within the highly conserved A–D elements reported for MKase (Potter et al. 1997). In addition, none of these mutations affect charged amino acids that might be expected to act in enzyme catalysis and/or charge transfer. Miziorko and colleagues have convincingly demonstrated that lysine 13 and glutamate 193 are involved in binding of MgATP in the MKase active site, whereas aspartate 204 is involved in enzyme catalysis (Potter and Miziorko 1997; Potter et al. 1997). None of our inherited mutations involve these residues. Sequence comparison of amino acid residues 240–270 of the MKase coding sequence—a mutational cluster suggested by our work—against other available sequences in the protein-sequence databases reveal that this region is well conserved only in MKase from other species and is not identified in substantive form in any other known proteins.

Preliminary attempts to correlate phenotype with genotype in MKD have not been satisfactory. As in other disorders, the expressed recombinant protein with the least residual activity does not necessarily occur in the most-severely affected patient. One of the least-active proteins identified in our work was the L264F allele, and the three siblings who carry this allele are more mildly affected than the other three patients in whom mutations were identified. Additional substrate-affinity analyses of each mutant protein may provide further insight. In addition, MKase is a dimeric protein, and all but two MKD patients analyzed are compound heterozygotes. It is difficult to predict the effect of compound mutations when each is expressed and analyzed as a single homozygous allele. Although all mutations identified thus far in MKD appear to cluster between amino acids 243 and 334, residing in the C-terminal portion of the MKase amino acid sequence, there is no single prevalent allele. The identification of additional mutations responsible for MKD, and determination of the exact effect of these mutations on enzyme folding and catalytic activity, may provide novel insight into structure/function relationships in MKase.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

ExPASy Molecular Biology Server (Enzyme Commission), http://www.expasy.ch/ (for E.C.2.7.1.36)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for MKase [MIM 251170])

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